EXHIBIT B (PART 1 OF 2)

CHAPTER III

GENETICS AND PHYSIOLOGY OF STARCH DEVELOPMENT

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I. Introduction

Starch, a common constituent of higher plants, is the major form in which carbohydrates are stored. Starch in chloroplasts is transitory and accumulates during the light period and is utilized during the dark. Storage starch accumulates in reserve organs during one phase of the plant's life cycle and is utilized at another time. Starches from reserve organs of many plants are important in commerce.

The pathway of starch synthesis is complex and not completely understood. Although gross starch structure is similar in various species, variation in granule structure and in starch fine structure is well documented and described elsewhere in this volume. Variation can be associated with plant species, cultivars of a species, environment in which a cultivar is grown, and genetic mutations.

This chapter first reviews nonmutant starch granule composition and development and then focuses on genetic mutants and how they have been useful in understanding the complexity of polysaccharide biosynthesis and development. Due to space limitation, attention is given only to a few of the plant species which are important sources of commercial starch production; the discussion will focus on maize (*Zea mays* L.) because of the many known endosperm mutants of maize which affect polysaccharide biosynthesis. Although developing maize kernels have been used for many of the investigations of starch biosynthesis, the information gained probably applies to other species, and these effects are illustrated whenever appropriate. As a result of this approach, it has been necessary to be selective in choosing examples to illustrate general trends in the genetics and physiology of starch development. Apology is given to other authors whose papers could also have been used to illustrate similar points.

No chapter can adequately cover all aspects of starch development, biosynthesis, and genetics. Readers wishing more detailed information should consult the books by Badenhuizen (*1*), Radley (*2*), and Banks and Greenwood (*3*); and review papers by Creech (*4*), Nelson (*5*), Preiss and Levi (*6*), Juliano (*7*), Marshall (*8*), Preiss (*285*), Preiss and Levi (*286*), and Banks and Muir (*287*).

II. Occurrence

1. General Distribution

Starch can be found in all organs of most higher plants (*1*, *9*). Organs containing starch granules include pollen, leaves, stems, woody tissues, roots, tubers, bulbs, rhizomes, fruits, flowers, and the pericarp, cotyledons, embryo, and endosperm of seeds. These organs range in chromosome number from the haploid pollen grain to the triploid endosperm, the main starch storing tissue of cereal grains.

In addition to higher plants, starch is found in mosses and ferns and in some protozoa, algae, and bacteria (*9*). Some algae, namely the Cyanophycae or blue-green algae (*10*, *11*), and many bacteria produce a reserve polysaccharide similar to the glycogen found in animals (*9*, *12*). Both starch and a water-soluble polysaccharide, similar to glycogen and termed phytoglycogen, occur in sweet corn and other maize genotypes (*13*). A glycogen-type polysaccharide also has been reported in the higher plant *Cecropia peltata* (*14*).

Badenhuizen (*9*) has classified starch producing species into two groups. In the first group, starch is formed in the cytosol of a cell, while in the second group, starch is formed within plastids.

2. Cytosolic Starch Formation

Starch granules are formed in the protozoa *Polytomella coeca* (*12*, *15*), but other species of protozoa produce amylopectin-type polysaccharides, glycogen, or laminaran (*12*, *15*).

The red algae, Rhodophyceae, produce a granular polysaccharide called Floridean starch on particles outside the chloroplasts. In many of its properties, this starch resembles the amylopectin of higher plants, but in other properties, it is intermediate between amylopectin and glycogen. Floridean starch contains no amylose (*10*, *16*). Free polysaccharide granules are also produced in the Dinophyceae, but the chemical nature remains uncertain (*10*).

Starchlike substances are produced in several species of bacteria (*9*, *12*). For example, *Escherichia coli* produces a linear polyglucan (*12*, *17*). *Corynebacterium diphtheriae* produces a starchlike material, and *Clostridium butyricum* produces a polyglucan with some branching (*12*). *Neisseria perflava* produces a

polyglucan intermediate in structure between amylopectin and glycogen (*17*); however, recent work shows that the structure more closely approaches that of glycogen (*18*).

3. Starch Formed in Plastids

Starch is formed in chloroplasts of moss, fern, and green algae (*9*). Chlorophyceae (green algae) starch is similar to that of higher plants, and several species have been used in studies of starch biosynthesis (*10*, *16*). Other classes of algae which produce starch are Prasinophyceae (*10*, *19*) and Cryptophyceae (*19*, *20*).

In the plastids of higher plants, starch granules can be classified as transitory or reserve (*1*). Transitory starch granules accumulate for only a short period of time before they are degraded. Starch formed in leaf chloroplasts during the day, which is subsequently hydrolyzed and transported to other plant parts at night in the form of simple sugar, is an example of transitory starch. Transitory starch is also formed in lily (*Lilium longiflorum*) pollen during germination of the pollen grains (*21*). Transitory and reserve starch granules can be differentiated by the fact that transitory starch granules lack the species specific shape associated with reserve starch granules. Furthermore, when exogenous sugar is supplied, the number, but not the size, of granules in a chloroplast will increase while the reverse occurs in amyloplasts (*1*).

Reserve starch is usually formed in amyloplasts, although it is occasionally formed in chloroamyloplasts. These are chloroplasts which have lost their lamellar structure and subsequently start producing fairly large reserve starch granules (*1*). Chloroamyloplasts form starch independent of photosynthesis. They have been described in tobacco (*Nicotiana tabacum* L.) leaves, *Aloe* leaves and flowers, central pith of potato (*Solanum tuberosum*) fruit, *Pellionia* and *Dieffenbachia* stems, and other tissues (*1*, *9*). Such sources of reserve starch are insignificant, however, when compared to the reserve starch formed in roots, tubers, and seeds.

III. Cellular Developmental Gradients

To properly evaluate data relating to reserve starch development and composition, cellular development of tissues in which this starch is formed must be appreciated. Enlarging potato tubers (*22*) and endosperms of developing maize (*23–27*), rice (*Oryza sativa* L.) (*7*), sorghum (*Sorghum bicolor* (L.) Moench) (*28–30*), wheat (*Triticum aestivum* L.) (*31*, *32*), rye (*Secale cereale* L.) (*33*), triticale (X *Triticosecale* Wittmack) (*34*), and barley (*Hordeum vulgare* L.) (*35*) kernels are composed of a population of cells of varying physiological ages.

In maize kernels, the basal endosperm cells begin starch biosynthesis late in development and contain small starch granules (*23*, *26*, *27*, *36*). Peripheral

maize endosperm cells, which are the last to develop, also contain small starch granules (*24, 26, 27, 36*). Thus, a major gradient of cell maturity from the basal endosperm to the central endosperm and a minor gradient from the peripheral cells adjacent to the aleurone layer inward exist in *normal* (nonmutant) maize endosperm. A similar cellular developmental gradient occurs in sorghum (*28–30*).

In barley, starch formation begins at the apex of the grain and around the suture across the central region (*35*). Deposition occurs last in the youngest cells near the aleurone layer (*35*). Related gradients occur in rice (*7*), rye (*33*), triticale (*34*), and wheat (*31, 37*).

Since all endosperm cells are not the same age, the physiologically younger cells may undergo the same developmental changes in starch biosynthesis as older cells, but at a later time in grain or kernel development. Shannon (*38*) divided 30-day-old *normal* maize kernels into seven endosperm zones and found that the sugar and starch composition of the lower zone corresponds to that found in whole endosperms 8, 10, and 12 days post-pollination, while the carbohydrate composition of upper zones is similar to that in the kernels 22–28 days post-pollination. When starch granules from 36-day-old *normal* maize kernels were separated into different size classes, a decline in apparent amylose percentage with decreasing granule size was observed, which reflected the characteristics of unfractionated starch isolated from endosperms earlier in kernel development (*39*). Although variations in granule size occur throughout the endosperm, starch granules within a given cell of *normal* maize endosperm are similar in size (*25, 36*).

The existence of cellular developmental gradients has two important ramifications when studying the genetics and physiology of starch development. First, evaluations of developing tissue, using whole tissue homogenates are based on polysaccharides and enzymes isolated from cells of differing physiological age. Thus, such whole tissue data represents only an average stage of cellular development at the date of sampling. Second, tissue that does not reach maturity because of environmental or other reasons will differ in composition from mature tissue, and variation in starch composition can occur between samples.

As tissues storing reserve starch develop and the cells fill with starch granules, the starch concentration, expressed as a percentage of tissue weight, increases. For example, the starch content of potatoes increases from 5 to 18% of the fresh weight as tuber size increases from 0–1 cm to 10–11 cm (*40*). In maize, numerous workers have demonstrated a similar increase with data reported by Wolf and co-workers (*41*) and Early (*42*) being typical. At 7–10 days post-pollination, starch comprises less than 10% of kernel weight. This percentage increases to 55–60% by 30–35 days, and then remains fairly constant until maturity. The starch content of barley kernels rises in a sigmoid pattern with time, and 95% is deposited between 11 and 28 days after ear emergence (*43*). Similar increases are observed in the reserve starch concentration in other species (*44–47*).

IV. Nonmutant Starch Granule Polysaccharide Composition

1. Polysaccharide Components

Nonmutant or *normal* reserve and transitory starch granules are composed primarily of amylose and amylopectin. Amylose is essentially a linear polymer consisting of (1→4)-linked α-D-glucopyranosyl units. Amylopectin is a branched polymer of α-D-glucopyranosyl units primarily linked by (1→4) bonds with branches resulting from (1→6) linkages (*48*, *This Volume*, Chap. VI). Properties of these two major starch components are summarized in Table I.

To determine the relative amounts of amylose and amylopectin in starch and the properties of these components, starch granules must first be isolated and purified from the plant species to be studied (*3*, *49*). Fractionation of the starch into its components can be achieved through two basic methods involving either selective leaching of the granules or complete granule dispersion (*3*, *49*, *50*). Methods based on granule dispersion are more satisfactory (*50*). Fractionation methods have been extensively reviewed (*3*, *50–53*, *This Volume*, Chap. VIII). Thus, only the basic aspects of these methods needed to establish a framework for discussing the starch composition of different species and genotypes will be presented. Methods for dispersing the granule have included autoclaving in water, solubilization in cold alkali, treatment with liquid ammonia, and solubilization in dimethyl sulfoxide, with the latter method preferred (*49*). Details are discussed in Chapter VIII.

Table I

Properties of the Amylose and Amylopectin Components of Starch[a]

Property	*Amylose*	*Amylopectin*
General structure	Essentially linear	Branched
Color with iodine	Dark blue	Purple
λ_{max} of iodine complex	~650 nm	~540 nm
Iodine affinity	19–20%	<1%
Average chain length (glucose residues)	100–10,000	20–30
Degree of polymerization (glucose residues)	100–10,000	10,000–100,000
Solubility in water	Variable	Soluble
Stability in aqueous solution	Retrogrades	Stable
Conversion to maltose by crystalline β-amylase	~70%	~55%

[a] Adapted from Marshall (*8*), Williams (*48*), and Radley (*2*).

Once dispersed, the differential iodine-binding properties of amylose and amylopectin (Table I) can be utilized to estimate the amount of linear polysaccharide present in the starch without fractionating the starch (*54*). Amylose can be determined either by measuring the absorbance of the starch–iodine complex (blue value procedure) and relating this absorbance to that obtained for amylose and amylopectin standards (*55–61*) or by the method of potentiometric iodine titration in which the amount (mg) of iodine bound per 100 mg of polysaccharide is determined and this amount is related to the amount bound by an amylose standard (*3, 54, 62, 63*). For nonmutant starches, these procedures give similar results (*54*); however, absolute results can vary with both procedures, depending on the iodine-binding properties of the amylose and amylopectin standards. Lansky and co-workers (*64*), for example, showed that iodine affinities for purified amyloses could range from 18.5 to 20.0% with some amylose subfractions having iodine affinities of 20.5–20.8%. Furthermore, the amylose content estimated by all of the procedures based on iodine complex formation should be considered "apparent amylose" (*65*); the occurrence of branched chain components with long external chains would result in an overestimation of the amylose content (*54, 66*). Likewise, the presence of short-chain-length amylose would cause the amylose content to be underestimated (*54*) because absorption of the starch–iodine complex is reduced when the average degree of polymerization is less than 100 (*67*). These limitations should be remembered when amylose percentages are presented.

Dispersed starch can be separated into the amylose and amylopectin components by adding a polar organic substance such as thymol or *n*-butanol to produce an insoluble amylose complex (see *This Volume*, Chap. VIII). This initial precipitate is usually purified by solubilizing the complex and recomplexing it. Amylopectin may be recovered from the initial supernatant by freeze-drying or by precipitation with alcohol (*3, 49, 50*). Alternatively, the amylopectin component can be removed first from the dispersion by high-speed centrifugation followed by the addition of a polar organic substance to precipitate the amylose from the supernatant (*68*). Recently, dispersed starch was fractionated using Sepharose column chromatography (*69, 70, 71*). All these procedures will permit quantitative estimation of the amount of amylose in the starch.

Amylose and amylopectin isolated following fractionation consist of a population of molecules that vary in their degree of polymerization (Table I). For example, amylose can be subfractionated into a graded series of molecular sizes (*64, 72, 73*); the amylopectin fraction also has a broad distribution of molecular weights (*74, 75*). In addition to heterogeneity of molecular sizes, amylose also appears to consist of a mixture of both linear and slightly branched chains, the proportions of which may vary with the source of the starch and with the maturity of the source (*3*). In short, both fractions are both polymolecular and polydisperse.

From this work it can be concluded that starches cannot be divided sharply into amylose and amylopectin, but that the two major fractions blend into each other through intermediate fractions. The presence of intermediate polysaccharides in the starch granule is apparent from the Sepharose elution profile of *normal* maize starch when compared to the profile of a mixture of purified amylose and amylopectin (*69, 71*). Based on indirect evidence from iodine affinities, Lansky and co-workers (*64*) suggested that 5–7% of *normal* maize starch consists of material intermediate between the strictly linear and highly branched fractions. Subsequently, several "non-amylopectin" types of branched polysaccharides have been recovered by various modifications of the previously described fractionation procedures. For example, Erlander and co-workers (*76*) recovered a low-molecular-weight component from the supernatant following amylose precipitation with thymol and removal of amylopectin by centrifugation. The polysaccharide remaining in the supernatant had a β-amylolysis limit and degree of branching similar to that of amylopectin. Perlin (*68*) obtained an intermediate component following removal of amylopectin by centrifugation and precipitation of amylose with amyl alcohol. The polysaccharide remaining in the supernatant was more highly branched than amylopectin, based on reduced β-amylolysis limits, and was of lower molecular weight. A related highly branched polysaccharide with viscosity similar to amylopectin was recovered from the supernatant following recomplexing the amylose fraction of starch from potato tuber, rubber (*Havea brasiliensis*) seed, barley kernels, and oat (*Avena sativa* L.) kernels (*77, 78*). A "loosely" branched polysaccharide related to amylopectin, but with greater average chain lengths and higher β-amylolysis limits, was recovered from rye and wheat starches (*78*) and from *normal* maize starch (*79*). 'Hinoat' oat starch was found to contain 26% of an intermediate molecular weight, branched starch component following Sepharose 2B gel filtration chromatography, while wheat starch contained 10% of a similar fraction (*80*).

Another polysaccharide reported in small amounts in starch of nonmutant rye (*78*), wheat (*78*), and maize (*81*) is short-chain-length amylose. In *normal* maize starch, this linear polysaccharide has an average chain length of 58 (*81*).

2. Species and Cultivar Effects on Granule Composition

The amylose concentration in nonmutant reserve starch of higher plants varies, depending upon the species and cultivar from which the starch is isolated. Deatherage and co-workers (*82*) analyzed starch from 51 species and reported a range of 11–37% amylose. A summary of data from the literature for 23 species indicates a range of 11–35% amylose (*48*). Starches of six species of legumes investigated had amylose contents which varied from 29% to 37% (*83*).

Almost as much variation for amylose percentage has been observed among cultivars of a single species, as observed among species. For example, amylose

percentage of starch ranges from 20% to 36% for maize (399 cultivars) (*82*, *84*), from 18% to 23% for potatoes (493 cultivars) (*85*), from 21% to 35% for sorghum (284 cultivars) (*82*, *86*, *87*), from 17% to 29% for wheat (167 cultivars) (*82*, *88*), from 11% to 26% for barley (61 cultivars including 5 genetic lines) (*89*, *90*), from 8% to 37% for rice (74 cultivars) (*91–94*), and from 34% to 37% for eight cultivars of peas (*Pisum sativum* L.) (*46*, *82*). Because of the variation in amylose concentration among species and among cultivars within a species, no average amylose percentage will be meaningful for nonmutant starches per se or for nonmutant starches of a given species. However, all nonmutant starches can be characterized as having more amylopectin than amylose.

Species and/or cultivar differences also are observed in other starch properties and in the properties of isolated amylose and amylopectin. To illustrate, purified amylose samples have been shown to differ in β-amylolysis limit and average degree of polymerization (*3*, *46*, *94*). Purified amylopectin samples also have been shown to differ in β-amylolysis limit, average length of unit chain, and viscosity (*3*, *46*, *48*, *94*, *95*).

3. Developmental Changes in Granule Composition

Increased amylose concentrations have been observed with increasing age of the tissue from which the starch was isolated for various plant species. Several authors (*39*, *41*, *96–98*) have reported increased amylose concentrations in maize endosperm during kernel development. For example, Tsai and co-workers (*98*) reported an amylose increase from 9% to 27% from 8 to 28 days post-pollination. The amylose concentration in potato starch increases from 12% in 0–1 cm tubers to 20% in 15–16 cm tubers (*40*). In starch from cassava (*Manihot utilissima*) roots harvested at various maturities, significant variation in amylose concentration (16–17%) has been observed; however, the net increase from 5 to 9 months of age amounts to only 0.3% (*99*). In starch of developing rice grains, amylose increases from 23% to 27% in cultivar 'IR8' from 4 to 39 days post-pollination (*100*) and from 30% to 37% from 3 days post-pollination to maturity in cultivar 'IR28', with 41% observed 7 days post-pollination (*44*). Amylose concentration has been shown to increase in developing wheat endosperms by various workers (*45*, *101–105*); however, the amount of increase varies with the initial sampling date and the cultivar examined. In starch of developing barley kernels, amylose concentration increases from 16% to 28% from 9 to 46 days after anthesis (*106*), from 13% to 25% and from 14% to 26% for two cultivars during a 12-week period (*107*), and from 14% to 22% from 14 to 30 days after ear emergence, with the concentration remaining constant from 30 days until maturity (*43*). The amylose concentration in smooth-seeded pea starch increases from 15% in 2–6 mm peas to 37% in 11–12 mm peas (*46*). Developmental differences also are observed in other starch properties and in the properties of isolated amylose and amylopectin (*40*, *46*, *99*, *100*, *103–105*).

Similar increases in amylose concentration are observed as a function of increasing granule size when granules from a developing tissue at a single stage of development are separated into various size classes (Table II). Since the smaller granules have lower amylose percentages similar to those of nonseparated starch granules from younger tissue, the smaller granules presumedly are isolated from the physiologically younger cells present in the developing tissue (see Section III). This effect of granule size on amylose concentration is not applicable to the small starch granules found in mature wheat and barley endosperms, since the small and large populations have similar properties (*45*, *108*, *109*). In barley and wheat, these small granules are formed late in the growth cycle and represent a second discrete population and not immature granules (*108*, *109*).

Because amylose concentration varies with maturity of the tissue, starches from tissues that do not reach maturity will be altered in their physicochemical properties from the corresponding mature starch.

4. Environmental Effects on Granule Composition

Growing conditions associated with different locations, years, planting dates, etc., can also affect the polysaccharide composition of nonmutant granules. Location and year of production and environmental conditions affect amylose concentration in rice (*91*, *110*) with milled samples of 'IR8' rice ranging from 27% to 33% amylose (*111*). The amylose content of 'Selkirk' wheat grown at four locations ranged from 23.5% to 24.7% (*88*) and of 'Katahdin' potatoes

Table II

Amylose Content of Starch Granules of Various Size Classes Isolated from Maize Endosperm 36 Days Post-Pollination and from Intermediate Size (5–6 cm) Potato Tubers

Maize[a]		*Potato*[b]	
Granule size, μm	*Amylose, %*	*Average granule size, μm*	*Amylose, %*
Unfractionated	25.4	28 (Unfractionated)	17.2
10 to 20	26.4	37	19.5
5 to 10	23.0	28	18.0
Less than 5	20.5	16	16.7
		10	16.0
		7	14.4

[a] Data from Boyer and co-workers (*39*).
[b] Data from Geddes and co-workers (*40*).

grown at three locations from 21% to 24% (*82*). The amylose concentration in starch from 30 samples of 'Compana' barley representing different environmental and cultural practices ranged from 19% to 23% (*89*). Limited variation was seen for amylose percentage in maize starch from plants grown for 3 years in each of eight states. Year effects ranged from 26.2% to 26.8% averaged over locations, and location effects ranged from 25.5% to 27.7% averaged over years (*112*). Although present, environmental effects are not as large as those associated with cultivars or cultivar maturity.

V. Nonmutant Starch Granule and Plastid Morphology

1. Description

Reserve starch granules in higher plant tissues develop in organelles called amyloplasts (*1*). An amyloplast may contain one starch granule, or it may contain several granules, depending on the plant species or genetic mutant. When only one granule is produced in an amyloplast, such as in wheat, potato, barley, maize, pea, and others, it is called a simple granule (*9*). When two or more granules occur in one amyloplast, they form the parts (granula) of one compound granule. Such granules are often rounded at first but become angular as they pack together within the plastid. The granula of the compound granule are separated by a narrow layer of stroma (*9*). Examples of species having compound granules include rice, oats, cassava, sweet potato (*Ipomoea batatas* L.), sago (*Metroxylon* sp.), and dasheen (*Colocasia esculenta*). Badenhuizen (*9*) terms semi-compound granules as those that are initially compound, but become united by the deposition of a common surrounding layer of starch. Starch granules from the bulb of *Scilla ovatifolia* are semi-compound (*9*). Goering and co-workers (*113, 114*) described the presence of large "starch chunks" in seeds of *Amarenthus retroflexus* (pigweed). The starch chunks are composed of many small granules cemented together with amorphous starch (*114*) and can be considered as semi-compound granules.

Wheat, rye, and barley produce two types of granules. The first granules produced in the endosperm cells develop into large lenticular granules (*9*). However, about two weeks after initiation of the first granules, additional small granules are produced within evaginations of the original amyloplasts, and then these separate from the original amyloplasts by constriction (*115*). The secondary granules are generally spherical and remain small. Although two basic size classes exist, no abrupt size change occurs, and some intermediate size granules are present (*32, 116*). In mature barley kernels, the large granules constitute about 90% of the total starch volume, but represent only 12% of the total number of granules (*117*). Large starch granules in 17 wheat flours averaged 12.5% of

the total granule number while accounting for 93.0% of the starch granule weight (*116*). Many of the large lenticular granules of wheat and barley have an equatorial groove or furrow (*118*, *119*). Buttrose (*118*) suggested that starch synthesizing enzymes may be concentrated within the equatorial groove.

2. Species and Cultivar Effects on Granule Morphology

Size and shape of reserve starch granules are extremely diverse and are species specific (*9*). This diversity is illustrated in photographs of starch granules from over 300 species and varieties (*120*). Microscopic characteristics of various starches are also summarized by Moss (*121*) and Kent (*122*). (See also *This Volume*, Chaps. XXIII and XXIV.) The scanning electron microscope (SEM) has been used to show the topography of starch granules. Hall and Sayre published SEM pictures of various root and tuber starches (*123*), cereal starches (*119*), and 16 other miscellaneous starches (*124*). (See also *This Volume*, Chap. XXIV.)

Smaller granules are often found in tissues of species producing compound granules such as rice (*119*), malanga (*Xanthosoma sagittifolium*) (*123*), and cowcockle (*Saponaria vaccaria* L.) (*124*). As noted earlier, the secondary granules in barley, wheat, and rye remain small, with most less than 10 μm in diameter (*122*). On the other extreme, large granules in potato tubers can exceed 120 μm in diameter (*123*). Starch granules from most species are non-uniform in size. The amount of this variation can be seen, for example, by examining granule size distributions for wheat (*125*), rye (*125*), triticale (*125*), potato (*40*), barley (*43*), maize (*39*, *41*), and dropwort (*Filipendula vulgaris*) (*126*).

Differences in average starch granule size in cultivars from a single species also have been reported. For example, average starch granule diameter ranged from 8.2 to 17.5 μm in 12 sorghum cultivars (*86*); from 17.8 to 25.6 μm in six triticale cultivars (*125*); and from 3.8 to 5.7 μm in 10 rice cultivars (*94*).

In addition to having an effect on amylose percentage (Section IV), varying environmental conditions also affected average starch granule diameter. Data on average starch granule size for rice cultivars grown in two different seasons (*92*) and dropwort grown at varying fertility levels (*126*) illustrate this effect.

In contrast to the species specific shape and size of reserve starch granules, transitory starch granules in chloroplasts appear similar in all species (*9*). In chloroplasts, the assimilatory starch granules are very small and disc-shaped (*1*).

3. Developmental Changes in Average Starch Granule Size

As tissues storing reserve starch mature, starch (Section III) and amylose (Section IV) concentrations increase. Similarly, average starch granule size in-

creases with increasing age of the storage tissues. Such increases have been documented in maize (*39, 41*) and rice (*100*) endosperm, in potato tubers (*40*), and in pea cotyledons (*46*). This trend does not apply to average starch granule size in barley, wheat, and rye where a second population consisting of a large number of small granules are formed late in development. In kernels of these species, average granule size initially increases; however, as the small granules are formed, average granule size decreases (*43, 45*). In barley the maximum average granule diameter of 10.5 μm was observed 16 days after ear emergence (*43*).

4. Formation and Enlargement of Nonmutant Granules

Plant cells have several types of plastids, such as proplastids, chloroplasts, chloroamyloplasts, chromoplasts, and amyloplasts, depending on the species and tissues. Badenhuizen (*1*) contends that, although certain plastids do not form starch under natural conditions, they all can be induced to form starch by floating tissue pieces on a sugar solution. Although starch can be produced in a variety of plastids when supplied with sugar, chloroplasts and amyloplasts are the primary sites of starch accumulation in nature. Transitory starch is produced in chloroplasts. In this case, small granules are formed between the lamellae of the chloroplasts during periods of excessive photosynthetic assimilate production (*1*). During extended light periods, the number of small granules in a chloroplast increases, but granule size remains relatively small (*1*). Starch granules in chloroplasts are partially degraded at night to supply sugars for translocation. The control of starch synthesis and degradation in chloroplasts will be discussed in Section VI.

Reserve or storage starch accumulates in specialized leucoplasts called amyloplasts and occasionally in chloroamyloplasts. Amyloplasts are organelles bounded by a double membrane which develop from proplastids. Duvick (*127*) studied early plastid and starch development in maize endosperm cells. He described small filaments which developed knobs in maize endosperm cells. Then, according to him, starch granules formed within the filament knobs. Based on more recent electron microscopic studies (*1, 118, 128*), the filaments observed in living cells by Duvick (*127*) apparently are proplastids developing into amyloplasts (knobbed filaments). Proplastids and young amyloplasts in fixed sections have very irregular shapes and likely assume various shapes (amoeboid) in the living cell (*128*).

The inner membrane of young amyloplasts from barley (*118*) and maize (*128, 129*) have been shown to be extensively invaginated to form tubuli, stroma lamellae, or vesicles. Badenhuizen (*129*) observed that starch granules are formed in the "pockets" provided by the lamellar structure. He suggested that

these pockets appear to be necessary for the initiation of starch granule formation, perhaps by promoting locally elevated concentrations of enzymes and substrates. The inner membrane of chloroplasts contain the specific translocators necessary for transfer of metabolites between the chloroplast stroma and the cytosol (*130*). It is assumed, based on the similarity between chloroplasts and amyloplasts, that the inner membrane of the amyloplasts also would function in regulation of metabolite transfer. Thus, the invaginations of the inner membrane noted above would effectively increase the area of the membrane and perhaps allow for more effective substrate transfer into amyloplasts (*118*).

The stroma (ground substance) of amyloplasts appears homogeneous by electron microscopic examination (*1, 128*). However, Badenhuizen (*9*) suggests that it contains inorganic and organic substances such as lipids, sugars, proteins, nucleotides, amino acids, nucleic acids, and inorganic ions. Liu and Shannon (*131*) confirmed the presence of many of these compounds plus various phosphorylated intermediates of gluconeogenesis in isolated starch granule preparations. (See also Section VI.) Presence of these various metabolites and proteins supports the suggestion that starch synthesis, not simply accumulation, occurs within the amyloplasts (*1*). Badenhuizen (*1, 9, 129*) has observed granular particles in the amyloplast stroma of tissue fixed in potassium permanganate. Although the granular structure observed may have been an artifact caused by the fixation procedure, it did show the presence of stroma material that accumulated in amyloplasts and then declined with the formation of the starch granule (*9*). The accumulation and decline of these particles also occurs during starch granule growth (*9*). Badenhuizen (*9*) called these particles coacervate droplets and concluded that they become attached to the periphery of the starch granule. Based on this observation, Badenhuizen (*1, 9*) concluded that starch molecules are produced in the amyloplast stroma and then the completed molecules become part of the growing starch granule. Shannon and co-workers (*132*) exposed maize plants to $^{14}CO_2$ and determined the distribution of ^{14}C in the amylose and amylopectin components of starch 1–6 h later. They found that the specific activity (^{14}C/mg of polysaccharide) of amylose and amylopectin increased at a similar rate, and that the radioactivity was distributed throughout the polysaccharide molecules. They concluded that these data supported Badenhuizen's (*1, 9*) suggestion that starch molecules are completely synthesized in the amyloplast stroma and then are deposited on the granule surface. Once the polysaccharides are part of the granule, there was no evidence of subsequent conversion of amylose to amylopectin (*132*). This is in contrast to conclusions drawn from long-term ^{14}C labeling studies of wheat starch (*133, 134*). In these studies, amylose appeared to be synthesized first, and then transformed into amylopectin. These differences may be due to the different species used or to the widely different sampling times.

VI. Polysaccharide Biosynthesis (see also Chapter IV, Section V)

1. Enzymology

Enzymes responsible for the synthesis of transitory starch in leaves and storage starch in seeds, tubers, etc., are generally considered to be the same in both types of tissues (*1*). Chloroplast starch is synthesized and accumulates during the light period when photosynthetic carbon fixation exceeds the demand for the assimilates by the plant, and it is hydrolyzed at night or when the assimilate demand exceeds current carbon fixation. Thus, synthesis and degradation or mobilization of transitory starch in chloroplasts are finely regulated. In storage tissues, starch synthesis is the predominant function of the amyloplast enzymes during tissue development. Thus, it is likely that the activity of amyloplast enzymes may be regulated by a mechanism different from that in chloroplasts.

Several pathways of starch synthesis have been proposed (*135–137*). Most of the evidence for these pathways comes from studies of isolated enzymes. With such *in vitro* studies, cellular compartmentation is destroyed, and one can only speculate as to the specific *in vivo* pathway of starch synthesis and the possible effector compounds which may be regulating synthesis. In spite of this shortcoming, such proposed pathways are important in focusing the investigator's attention on areas of research needed in the future. One such pathway is given in Figure 1. All the enzymes in this pathway have been measured in extracts from maize endosperm and other starch storing tissues.

For purposes of this discussion, sucrose is considered as the primary substrate for starch biosynthesis. In storage tissues, the UDPG formed from the action of sucrose synthase (*135, 138*) (Fig. 1, enzyme 6) can be utilized directly for starch synthesis by granule-bound starch synthase (Fig. 1, enzyme 9), or it can be converted to D-glucose 1-phosphate (G-1-P) by UDPG pyrophosphorylase (Fig. 1, enzyme 7) (*135*). Evidence that sucrose synthase may be significant in the production of substrates for starch synthesis *in vivo* comes from the observation that the maize endosperm mutant *shrunken* (*sh*), which causes a 40% reduction in kernel starch relative to *normal*, also causes a reduction in sucrose synthase activity (*139*). G-1-P can also be produced from sucrose via the combined action of invertase, hexokinase, phosphoglucoisomerase (for the D-fructose moiety), and phosphoglucomutase (Fig. 1, enzymes 1,2,4,5, respectively). The G-1-P produced by either mechanism can function as a substrate for phosphorylase (Fig. 1, enzyme 11) (*138, 140*). G-1-P also can serve as substrate for ADPG pyrophosphorylase (Fig. 1, enzyme 8) which yields ADPG. ADPG can serve as substrate for both granule bound and soluble starch synthase (Fig. 1, enzymes 9 and 10) (*141*). Thus, phosphorylase, starch-granule-bound starch synthase, and

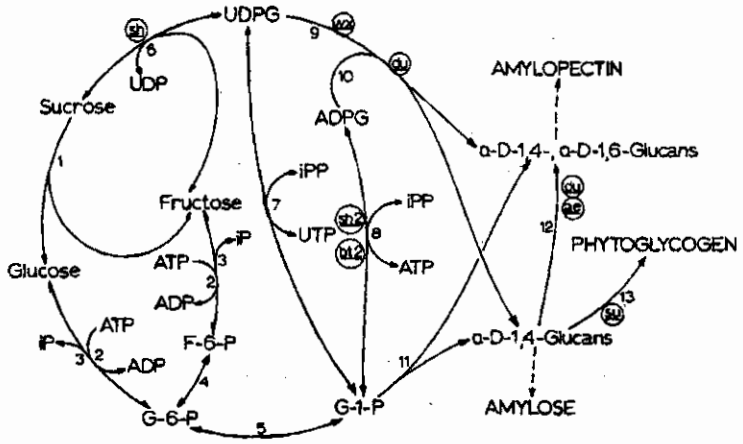

FIG. 1.—Hypothetical scheme for the conversion of sucrose to amylose, amylopectin, and phytoglycogen in *Zea mays* L. kernels [adapted from Boyer and Shannon (*296*)]. The endosperm mutant symbols are positioned at the enzyme sites shown to be affected by the different mutations (see Table X). Enzymes indicated by the small numbers are as follows: 1 = invertase (EC 3.2.1.26, β-D-fructofuranosidase); 2 = hexokinase (EC 2.7.1.1); 3 = hexose-6-phosphatase (EC 3.1.3.9, glucose-6-phosphatase); 4 = glucosephosphate isomerase (EC 5.3.1.9); 5 = phosphoglucomutase (EC 2.7.5.1); 6 = sucrose synthase (EC 2.4.1.13); 7 = UDP-glucose pyrophosphorylase (EC 2.7.7.9, glucose-1-phosphate uridylyltransferase); 8 = ADP-glucose pyrophosphorylase (EC 2.7.7.27, glucose-1-phosphate adenylyltransferase); 9 = starch granule bound starch synthase (EC 2.4.1.21); 10 = soluble starch synthase; 11 = starch phosphorylase (EC 2.4.1.1); 12 = Q-enzyme (EC 2.4.1.18, 1,4-α-D-glucan branching enzyme); 13 = phytoglycogen branching enzyme.

soluble starch synthase are all capable of catalyzing the *in vitro* synthesis of (1→4)-α-D-glucosidic bonds. The question may be asked whether one of these is the predominant enzyme for starch synthesis *in vivo*.

Prior to about 1960, phosphorylase was the only known enzyme capable of producing (1→4)-α-D-glucan polymers (*140, 142*). Multiple forms of phosphorylase have been reported in maize (*143, 144*), potatoes (*145*), *Vicia faba* (*146–148*), *Pisum sativum* (*149*), and *Phaseolus vulgaris* (*146*). Certain of these phosphorylase enzymes increase during the period of active starch synthesis and deposition, and it has been suggested that these phosphorylases may play an important role in starch synthesis (*143–148*). However, others have discounted phosphorylase as a starch synthetic enzyme because of the unfavorable inorganic phosphate (P_i) to G-1-P ratio in plant cells (*6*). Starch synthesis from G-1-P by phosphorylase requires a relatively high concentration of G-1-P. Although it has been shown that the concentration of P_i is many fold higher than that of G-1-P in whole cell homogenates (*131*), some investigators contend that at the site of synthesis, the amyloplast stroma, the P_i to G-1-P ratio may favor synthesis (*1, 3, 143*). In immature maize endosperm, the concentration of P_i is over 100 times

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higher than that of G-1-P (*131, 150*). Likewise, a recent analysis of components associated with starch granules isolated nonaqueously from maize kernels showed a similar very high P_i to G-1-P ratio (*131, 150*). Thus, in maize kernels, it has been concluded that *in vivo* starch synthesis by phosphorylase is highly unlikely (*131, 150*).

The discovery in 1957 of the role of nucleoside diphosphate sugars in carbohydrate metabolism stimulated greater interest in polysaccharide biosynthesis. Much of the early work came from L. F. Leloir's laboratory, and he summarized many of his contributions to the advancement of carbohydrate metabolism when he accepted the 1970 Nobel Prize in chemistry (*141*). Many studies of the starch synthases have been reported and recently reviewed (*6, 138, 151, 152, 286*).

Starch synthases from photosynthetic tissues and the soluble starch synthases from storage tissues are virtually specific for ADPG (*153*). Starch granule-bound starch synthases from storage tissues may utilize both ADPG and UDPG, but ADPG appears to be the preferred substrate (*154*). Tsai (*155, 156*) reported that the starch granule-bound starch synthase more actively transfers the D-glucose from UDPG and ADPG to amylose, while the soluble starch synthases preferentially transfer glucose to amylopectin *in vitro*.

The *waxy* (*wx*) mutants of rice (*157*) and maize (*156, 158, 159*) have only very low starch granule-bound starch synthase activities. Akatsuka and Nelson (*160*) suggested that the normal allele at the *wx* locus is either the structural gene for the starch granule-bound starch synthase or that it specifies receptor sites on the starch granule to which the enzyme binds. Recently, Nelson and co-workers (*159*) reported the presence of a minor starch synthase activity in *wx* starch granules. They speculated that a similar minor synthase activity is present in nonmutant starch granules also. The *wx* mutants which accumulate starch with approximately 100% amylopectin contain two soluble ADPG starch synthases (*156, 157, 161*).

Although granule bound starch synthases from storage tissues can utilize UDPG *in vitro*, it appears that ADPG is the predominant substrate for starch synthesis *in vivo*. Evidence for this comes from the observation that the maize endosperm mutants *shrunken-2* (*sh2*) and *brittle-2* (*bt2*) are lacking (*162*) or show very low (*163, 288*) ADPG pyrophosphorylase activity. However, *sh2* endosperms have normal levels of UDPG and CDPG pyrophosphorylases (*162, 164*). The fact that *sh2* mutant kernels are very low in starch and high in sucrose relative to *normal* kernels underscores the importance of ADPG as the primary substrate for starch synthesis (*6*).

Both phosphorylases and starch synthases catalyze the addition of D-glucose to the nonreducing ends of primer maltooligosaccharide molecules through (1→4)-α-D-glucosidic bonds. Thus, branching enzyme activity is necessary to produce amylopectin. Haworth and co-workers (*165*) first reported such a transglycosylase, Q-enzyme (Fig. 1, enzyme 12), in 1944. This enzyme produces

(1→6)-α-D branches by cleaving a fragment from the linear chain and transferring it to the number six position of a glucose residue of the growing polymer (*152, 166, 167*). Multiple branching enzyme activities have been reported in spinach leaf (*168*), *smooth-seeded pea* (*Pisum sativum* L.) (*289*), and maize endosperm (*169*) extracts. When separated by DEAE-cellulose chromatography, one peak of branching activity is coincident with one of the soluble starch synthase activity peaks (*169*). This starch synthase–branching enzyme mixture, in the presence of high salt (0.5 *M* sodium citrate), is capable of catalyzing the synthesis of an amylopectin-like polyglucan in the absence of added maltooligosaccharide primer. Schiefer and co-workers (*170*) electrophoretically separated extracts from developing maize endosperm and identified several bands of starch synthase activity. Based on the iodine staining properties of the product, they concluded that certain starch synthases are bound to branching enzyme and others are free from branching enzyme. They suggested that, in the cell, starch synthases may exist in a free form or as a branching enzyme–starch synthase complex. The *ae* mutant, which accumulates starch with a higher amylose percentage than *normal*, also has more free starch synthase relative to the branching enzyme-starch synthase complex (*170*). Also, the *su* mutant, which produces the more highly branched polyglucan, phytoglycogen, produces a brown staining band indicative of the presence of a phytoglycogen branching enzyme–starch synthase complex (*170*). Boyer and Preiss (*171*) chromatographically separated branching enzymes from *normal* and *ae* and found that one of the branching enzyme fractions (fraction IIb) is absent in *ae*. In view of the possible presence of a branching enzyme–starch synthase complex *in situ* and the stimulatory effect of citrate on the polyglucan synthetic activity of this complex *in vitro*, Boyer and co-workers (*96*) suggested that citrate or some similar metabolite may stabilize the branching enzyme–starch synthase complex *in situ*.

Amylose and amylopectin appear to be synthesized simultaneously *in vivo*. Shannon and co-workers (*132*) found that the amylose and amylopectin components of starch from developing maize kernels harvested one to 36 h after exposure of the intact plant to $^{14}CO_2$ had similar specific radioactivities at each sampling time. Furthermore, the radioactivity was distributed throughout the amylose and amylopectin molecules. These data fit a model suggested by Badenhuizen (*1*) in which the polysaccharides are synthesized in the matrix of the amyloplast followed by crystallization of the completed molecules onto the starch granule. This study (*132*) supports the suggestion of Schiefer and co-workers (*170*) that a branching enzyme–starch synthase complex may exist *in situ;* if so, free amylose would not be an intermediate in the synthesis of amylopectin.

Other enzymes of possible significance in starch synthesis or degradation have been reported and reviewed by Marshall (*8*). A disproportionating enzyme, D-enzyme (EC 2.4.1.25), was first reported in potatoes (*172*) and has been reported

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in sweet corn. The significance of this enzyme in starch synthesis is unknown, but it may function in the mobilization of starch to phytoglycogen in the *su* mutant. Plant debranching enzyme (R-enzyme, EC 3.2.1.9) may also be functioning in starch mobilization to phytoglycogen in *su* (*173*). However, in *normal* cereal kernels, it functions during germination (*8*), and it is not known to have a starch synthetic function. Erlander (*174*) proposed a mechanism for the synthesis of starch from glycogen, but Marshall (*8*) considers it an unlikely scheme. Amylosucrase (EC 2.4.1.4) is a bacterial (*Neisseria perflava*) enzyme that brings about the synthesis of a high-molecular-weight glycogen or amylopectin-type α-glucan from sucrose (*17*). This enzyme catalyzes the production of α-glucans from sucrose, with no indication of mediation by UDPG, ADPG, or G-1-P (*18*). The glycogen-like product of amylosucrase indicates either that the enzyme catalyzes the formation of both (1→4)-α-D and (1→6)-α-D linkages, or that it exists as a polymerizing–branching enzyme complex (*18*). Okada and Hehre (*18*) pointed out that the extent of *in vitro* D-glucose polymerization catalyzed by amylosucrase (250 parts D-glucose transferred for each part of preformed polysaccharide) is much greater than the D-glucose transfers reportedly obtained by the combined sucrose synthase–starch synthase system from rice grains (1 part transferred per 100 parts precursor) or sweet corn and beans (1 part transferred per 5000 parts precursor). Although amylosucrase has not been found in higher plants, Okada and Hehre (*18*) wonder whether the sucrose to starch conversion process in intact plants has been adequately defined. Additional studies are needed to establish whether an amylosucrase-type enzyme is functioning in the *in vivo* synthesis of starch in higher plants.

2. Compartmentation and Regulation of Starch Synthesis and Degradation in Chloroplasts

Photosynthesis occurs in organelles called chloroplasts. Chloroplasts develop from proplastids and are bounded by a double membrane (*1*). The outer membrane is freely permeable to small molecules, but the inner membrane is a functional barrier between the chloroplast stroma and the cytosol (*130*). The inner membrane is the site of specific metabolic transport systems. Specific translocators described in chloroplast envelopes are the phosphate translocator, dicarboxylate translocator (*175*), and an ATP translocator (*176*). The presence of two amino acid translocators in chloroplast membranes also has been suggested (*177*). The chloroplast membrane is essentially impermeable to free sugars such as D-glucose, D-fructose, and sucrose. Hexose phosphates and pentose phosphates cross the membrane very slowly (*178, 179*). Dihydroxyacetone phosphate (DHAP), 3-phosphoglycerate (3-PGA), and P_i move in and out freely via the phosphate translocator (*175*). The dicarboxylate translocator facilitates the rapid exchange of dicarboxylates such as malate, oxaloacetate, succinate, α-ketogluta-

rate, and fumarate, and related amino acids such as asparate and glutamate. ATP can cross the membrane by means of the ATP translocator, but this is not the major system for the transfer of energy from the chloroplast to the cytoplasm (*178–180*).

Chloroplasts which produce starch under natural conditions reduce carbon by the Calvin cycle of photosynthesis. The mesophyll cells of so called C_4 plants, such as maize, fix carbon by the C_4 pathway of photosynthesis (*181*), but they generally produce very little starch (*182, 183*). Rather, the four carbon acids produced in the mesophyll cells are transferred to the bundle sheath cells where they are decarboxylated and the resulting CO_2 is refixed by the Calvin cycle enzymes in the bundle sheath chloroplast (*181*). Bundle sheath chloroplasts of C_4 plants accumulate starch (*183*). In the Calvin cycle, the enzyme ribulose-bisphosphate carboxylase catalyzes the carboxylation and cleavage of ribulose 1,5-bisphosphate to yield two molecules of 3-PGA (*181*). The 3-PGA molecules thus produced by photosynthesis are (*a*) used as a substrate for the Calvin cycle, (*b*) unloaded from the chloroplasts via the phosphate translocator (*175*), and/or (*c*) utilized in the production of chloroplast starch. The triose phosphates transferred from the chloroplasts are used for the synthesis of sucrose in the cytoplasm by the combined activity of enzymes of gluconeogenesis and sucrose synthesis (*179*). Similar gluconeogenetic enzymes, components of the Calvin cycle, produce F-6-P which can then be converted by glucosephosphate isomerase into G-6-P, which in turn is converted to G-1-P by phosphoglucomutase. The G-1-P then can be utilized in the production of starch directly by phosphorylase, or more likely it is converted by ADPG pyrophosphorylase into ADPG, which is the substrate for the starch synthases. Although there is still disagreement among investigators as to the relative importance of the phosphorylases and starch synthases in the *in vivo* synthesis of starch in storage tissues, most researchers agree that in chloroplasts, D-glucose is polymerized by the bound and soluble starch synthases from ADPG and that phosphorylase functions in starch mobilization (*6*).

Chloroplast starch is degraded at night or during periods when assimilate demand exceeds current photosynthetic production (*6*). Mobilization of chloroplast starch involves enzymes which cleave the (1→4)-α-D-glucosidic bonds and the (1→6)-α-D-glucosidic branches. The released sugars or sugar phosphates (G-1-P) must then be converted to triose phosphates which can be transported to the cytosol via the phosphate translocator. Preiss and Levi (*6*) recently reviewed the various hydrolyses possibly involved in starch degradation and concluded that "little definitive work has been done on the localization or regulation of the enzyme of the first steps of starch degradation."

α-Amylase is generally accepted as one of the most important enzymes in the hydrolysis of storage starch granules, with β-amylase and phosphorylase being less important (*6*). Most recent studies indicate that phosphorylase is the primary

enzyme involved in hydrolysis of leaf starch (*184*). Levi and Priess (*184*) suggested that the small amounts of maltose found in pea chloroplasts during starch degradation may have been produced from G-1-P and D-glucose by maltose phosphorylase rather than from the action of α-amylase or β-amylase. Although phosphorylase appears to be responsible for cleavage of the (1→4)-α-D-glucosidic bonds of starch, there is no definitive information on enzymes responsible for hydrolysis of the (1→6)-α-D-linkages in amylopectin. It is assumed that one of the debranching enzymes is involved (*185*).

As noted earlier, fine control is necessary to regulate the synthesis and degradation of transitory starch in chloroplasts. Current evidence indicates that starch synthesis is regulated by the production of the substrate ADPG by ADPG pyrophosphorylase. Priess and co-workers have extensively studied the pyrophosphorylases from bacteria, green algae, chloroplasts, and storage tissues (*186–188, 285*). In all photosynthetic tissues tested, ADPG pyrophosphorylase was activated by glycolytic intermediates and inhibited by P_i. An early product of photosynthesis, 3-PGA is the most effective activator of ADPG pyrophosphorylase from green algae and leaves (*186*). Thus, it is generally accepted that ADPG pyrophosphorylase functions as a regulatory enzyme which responds to positive (3-PGA) or negative (P_i) allosteric effectors (*6, 184, 186–188*). During periods of excess carbon fixation, 3-PGA increases in the chloroplasts which stimulates the production of ADPG from ATP and G-1-P resulting in an increased synthesis of starch (*186*). In the dark, the photosynthetic production of 3-PGA ceases, the level of ADPG declines, and starch synthesis ceases (*186*). Also in the dark, P_i in chloroplasts increases 30–50%, and the pH of the chloroplasts' stroma declines. Preiss and Levi (*6*) suggested that the lower pH may enhance activity of certain starch hydrolases, and the increased P_i and lower ADPG concentrations may stimulate starch hydrolysis by phosphorylase. However, they (*6*) add that definitive studies on the regulation of chloroplast starch degradation are lacking.

3. Compartmentation and Regulation of Starch Synthesis in Amyloplasts

Amyloplasts are organelles specialized for the accumulation of starch in storage cells. They develop from proplastids, as do chloroplasts, and are bounded by double membranes (*1*). Amyloplasts develop into chloroplasts under certain conditions (*189*) and vice versa (*1*). Thus, it is assumed that the nature of the amyloplast envelope may be like that of the chloroplast with a similar membrane transport system (*190*). The inner membrane of young amyloplasts from barley (*118*) and maize (*128*) has been shown to be extensively invaginated to form tubuli, stroma lamellae, or vesicles. Buttrose (*118*) suggested that if the inner membrane is the one limiting uptake, the increased area resulting from the invaginations of the inner membrane would allow for more rapid uptake of

sugars or sugar phosphates into the amyloplast stroma. However, since we now know that chloroplast membranes are relatively impermeable to neutral sugars and hexose phosphates, it is more likely that carbohydrates may enter the amyloplasts as triose phosphates via the phosphate translocator (*131, 150, 190*).

Amyloplasts containing starch granules are extremely fragile, and attempts to aqueously isolate intact amyloplasts have been disappointing. Therefore, it has not been possible to directly study the membrane translocators in isolated amyloplasts. This problem has also made attempts to study the compartmentation of enzymes in amyloplasts very difficult. However, papers from Viswanathan's laboratory (*191–193*) reported the presence in isolated amyloplasts of all enzymes necessary to convert glucose and fructose to starch. Unfortunately, attempts to repeat Viswanathan's results in my laboratory were completely unsuccessful (Shannon, unpublished data). Williams and Duffus (*194*) isolated "amyloplasts" from barley endosperm using an aqueous media and studied the distribution of enzymes of carbohydrate metabolism and starch synthesis between the amyloplasts and the cytosol. They concluded that the production of G-1-P and ADPG from sucrose occurs in the cytosol. However, they presented no evidence that the isolated amyloplasts were intact and indeed contained the plastid stroma enzymes. Fishwick and Wright (*290*) were able to purify sufficient amyloplasts from potato (*Solarum tuberosum* L.) to characterize the lipids of the amyloplast envelope membrane. However, they pointed out that the yield of intact amyloplasts rarely exceeded 16%.

Because of the difficulty in isolating intact amyloplasts for studying enzyme compartmentation, we (*131, 150*) approached the question of what reactions of starch synthesis are occurring in amyloplasts by determining the metabolite composition of nonaqueously isolated "amyloplasts." For this work, maize endosperm slices were quick frozen and freeze dried, and the starch granules were isolated using glycerol and 3-chloro-1,2-propanediol (*150*). These granule preparations were relatively free of cytoplasmic and nuclear contamination, based on RNA and DNA content, respectively, but they contained the metabolites which are closely associated with the starch granules *in situ* and which become fixed to the granules during freeze drying. These components are thought to represent the *in situ* metabolites of the amyloplast. The starch granule preparation contained neutral sugars, P_i, intermediates of gluconeogenesis, organic acids, and amino acids, as well as adenosine and uridine nucleotides and nucleoside diphosphate sugars (Table III). Some of these constituents, such as the neutral sugars, malate and inorganic phosphate, accumulate to relatively high levels. Over 30% of the cellular malate and P_i and over 15% of the intermediates of gluconeogenesis from DHAP to G-1-P were recovered with the starch granules. The starch granule preparation was also relatively rich in the adenosine and uridine nucleotides. Approximately 10% of the cellular ADPG and UDPG were recovered in the starch granule preparation. The lower percentage of these two

Constituent	*nmole/m. starch*
Sucrose	62.50
Glucose	21.30
Fructose	11.40
G-1-P	0.10
G-6-P	4.44
F-6-P	0.93
FDP	0.24
3-PGA	0.82
DHAP	0.20
G-3-P	0.03
PEP	0.34
P_i	15.15
Citrate	0.85
Malate	17.39
Pyruvate	0.11

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Quantity and Percent of Various Cellular Constituents Associated with Nonaqueously Isolated Starch Granules[a]

Constituent	nmole/mg starch	%[b]	Constituent	nmole/mg starch	%[b]	Constituent	nmole/mg starch	%[b]
Sucrose	62.50	13	AMP	2.66	29	Lysine	1.21	21
Glucose	21.30	26	ADP	1.09	15	Histidine	0.25	25
Fructose	11.40	16	ATP	2.43	22	Arginine	T[c]	T
G-1-P	0.10	25	UMP	2.79	24	Aspartic acid + unknown	8.96	38
G-6-P	4.44	21	UDP	1.45	15	Threonine + serine	47.50	32
F-6-P	0.93	28	UTP	3.83	19	Glutamic acid	14.91	40
FDP	0.24	16	ADP-Glc	0.68	9	Proline	4.17	39
3-PGA	0.82	7	UDP-Glc	1.91	11	Cysteine	T	T
DHAP	0.20	27	NAD	0.80	10	Glycine	2.63	51
G-3-P	0.03	7	NADP	0.24	17	Alanine	28.11	43
PEP	0.34	19				Valine	2.48	32
P_i	15.15	34				Methionine	1.07	35
Citrate	0.85	6				Isoleucine	0.39	26
Malate	17.39	34				Leucine	0.57	27
Pyruvate	0.11	14				Tyrosine	0.48	29
						Phenylalanine	0.42	29

[a] Data adapted from Liu and Shannon (*131*).
[b] % of the total cellular constituents in the granule preparation.
[c] Trace amount.

primary substrates of starch synthesis in the amyloplasts may mean that their rate of utilization in starch synthesis is higher than their utilization in other pathways outside the amyloplasts. More than 20% of all free cellular amino acids were recovered in the starch granule preparation. Amounts of threonine plus serine, alanine, and glutamic acid were high in both the cellular and starch granule preparation, compared to amounts of the other free amino acids (Table III).

Based on the metabolite compartmentation in the glycerol-isolated starch granules, the mechanism of carbohydrate metabolism in corn endosperm cells given in Figure 2 was proposed (*131, 150*). In this scheme, the hexoses are converted to DHAP in the cytoplasm via glycolysis (Fig. 2, enzymes 2,4,14,16, and 17), the DHAP moves via the phosphate translocator into the amyloplast where it is converted into starch by the combined action of gluconeogenesis (Fig. 2, enzymes 17,16,15,4, and 5) and starch synthesis (Fig. 2, enzymes 7,8,9 and 10). Q-enzyme is also required for amylopectin production.

The high recovery of the cellular malate (34%) in the starch granule preparation may indicate the functioning of a dicarboxylate translocator in the amyloplast membrane. Citrate is excluded from chloroplasts (*175*) and also is

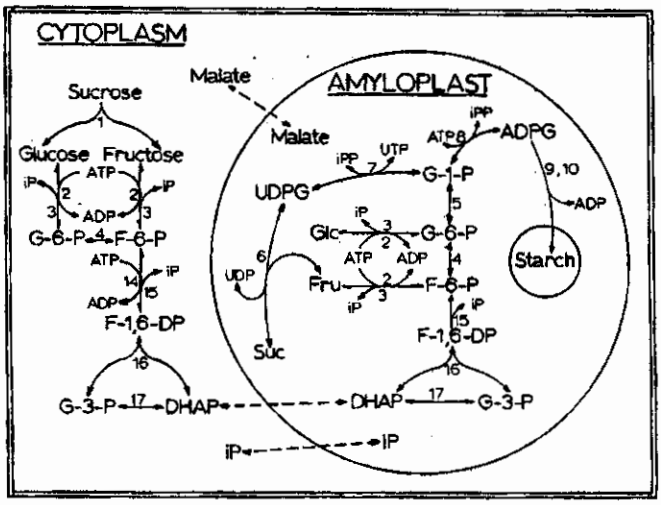

FIG. 2.—Proposed compartmentation of metabolites and enzymes of carbohydrate metabolism and starch synthesis between the cytoplasm and amyloplasts of maize endosperm cells. Adapted from Shannon (*297*). Enzymes indicated by the small numbers are as given in Figure 1 plus the following: 14 = 6-phosphofructokinase (EC 2.7.1.11); 15 = fructose-1,6-diphosphatase (EC 3.1.3.11, fructose-bisphosphatase); 16 = fructose-diphosphate aldolase (EC 4.1.2.13, fructose-bisphosphate aldolase); 17 = triosephosphate isomerase (EC 5.3.1.1).

excluded from amyloplasts, with only 6% of the total being recovered in the granule preparation (Table III).

Several conclusions can be drawn from an examination of the compartmentation of metabolites in the starch granule preparation. (a) The quantity of G-6-P is over 40 times greater than that of G-1-P. Thus, G-1-P is utilized almost as rapidly as it is produced, indicating that phosphoglucomutase (enzyme 5), the enzyme catalyzing the reversible interconversion of G-6-P and G-1-P, may be one of the rate limiting steps in starch synthesis. (b) The quantity of G-1-P is very low (0.1 nmoles/mg starch) compared to inorganic phosphate (15.2 nmoles/mg starch). Because the P_i to G-1-P ratio is over ten times higher than the equilibrium constant for phosphorylase, starch breakdown would be favored (*6*). Thus, these data do not support a starch synthetic function for phosphorylase in maize kernels. (c) The quantity of 3-PGA in the starch granule preparation is relatively low and represents only 7% of that in the whole cell. As noted earlier, 3-PGA is an allosteric effector compound which stimulates *in vitro* production of ADPG by chloroplast derived ADPG pyrophosphorylase (*186, 188*). The ADPG pyrophosphorylase (Fig. 2, enzyme 8) from maize endosperm is also stimulated by 3-PGA, but to a much lesser extent (*195*). From the results of this compartmentation study, it appears that 3-PGA is not functioning *in situ* as an effector metabolite for ADPG pyrophosphorylase in maize endosperm cells. (d) Citrate

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enhances gluconeogenesis by stimulating hexose diphosphatase (*187, 188*) (Fig. 2, enzyme 15); it has been shown to stimulate the *in vitro* activity of unprimed starch synthase (*161, 196*). Because malate rather than citrate accumulates in the amyloplasts (Table III), we suggest that, in the intact cell, malate may be functioning in the control of gluconeogenesis and in the stabilization of the starch synthase–branching enzyme complex. Boyer and Preiss (*197*) found that malate was 80% as effective as citrate in stimulating the *in vitro* activity of unprimed starch synthase.

VII. Mutant Effects

Maize is unique among higher plants relative to the number of mutants which have been identified and extensively examined. Several mutants effect the quantity and quality of carbohydrates in the triploid endosperm. Furthermore, these mutants often modify kernel development (*26, 27*), mature kernel phenotype (*198*), and starch granule morphology (*26, 71, 199*). The *shrunken* (*sh*), *shrunken-2* (*sh2*), *brittle* (*bt*), and *brittle-2* (*bt2*) mutants condition an accumulation of sugars at the expense of starch. The *shrunken-4* (*sh4*) mutant, which causes a reduction in starch accumulation, was originally thought to be a phosphorylase mutant (*200*), but it was later shown to affect the quantity of pyridoxal phosphate (*201*), thus, reducing the activities of several endosperm enzymes such as phosphorylase, which require pyridoxal phosphate. The *sh* mutant causes a reduction in sucrose synthase activity (*139*), while *sh2* and *bt2* each lack (*162*) or have very low levels of ADPG pyrophosphorylase (*163*). The genetic lesion associated with *bt* is unknown at this time.

Mutants affecting endosperm protein production include *opaque-2* (*o2*), *opaque-6* (*o6*), *opaque-7* (*o7*), *floury* (*fl*), *floury-2* (*fl2*), and *floury-3* (*fl3*). These mutants all cause a reduction in the prolamin (zein) fraction of storage proteins and a compensatory increase in albumin and globulin fractions (*5*). The mutant *soft starch* (*h*) causes a loose packing of starch in the endosperm cells, but has not been related to any major change in storage proteins (*202*), starch composition (*203*), or starch granule structure (*199*).

Because the primary focus of this section is mutant effects on polysaccharide composition, mutants in maize which cause changes from *normal* in amylose percentage and phytoglycogen production will be reviewed. The maize mutants in this group include *waxy* (*wx*), *amylose-extender* (*ae*), *sugary* (*su*), *sugary-2* (*su2*), and *dull* (*du*). These mutants alone and in various multiple mutant combinations have dramatic effects on kernel development, starch granule development and morphology, and polysaccharide composition (*4*). As will be pointed out subsequently, certain mutants cause the production of polysaccharides differing from standard amylose and amylopectin in molecular weight and degree of branching.

From 0 to 12 days post-pollination, little or no detectable differences are observed between *normal* and these mutants (except *su*) with respect to kernel and amyloplast development. The various mutant effects thus become expressed during the major period of starch accumulation. The mutant *su* differs from *normal* and the other mutants by initially producing compound granules (*27*, *128*).

Saussy (*26*) made an extensive survey of mutant effects on maize endosperm and starch granule development at 16 and 27 days post-pollination. '1A5125' versions of *normal*, *ae*, *du*, *su*, and *wx* singly and in double, triple, and quadruple combinations (except *su wx* and *ae su wx*) were studied. *Normal* and all mutant genotypes exhibited the major gradient of starch granule development from the kernel base (least mature) to the central crown region of cells (most mature) described in Section III. Two basic types of minor gradients of starch granule development were observed. The type I minor gradient is similar to that described for *normal*, with an increase in cellular maturity inward from the peripheral cells adjacent to the aleurone layer (*24*, *27*). The type II minor gradient is similar to type I along the peripheral endosperm and toward the interior for a few cell layers (variable with the genotype), but then an abrupt decrease in the volume of cellular inclusions occurs. These differences in minor gradients and other specific mutant effects will be noted in discussion of the mutants.

For convenience, the effects of the various mutants and mutant combinations including information on kernel phenotype, starch granule size and physical properties, WSP concentration, amylose percentage, and relative sizes and iodine binding capacity of polysaccharides following separation by Sepharose column chromatography are summarized in Tables IV, V, VI, VII, and VIII. Current information on the specific mutants singly and in combination and information on similar mutants in other species when such mutants are known is presented below.

1. Waxy

Waxy (*wx*) or *glutinous* (*gl*) loci have been identified in maize, sorghum, rice, barley, and Job's tears (*Coix lachryma-jobi*) (*209*, *210*). These mutants produce starch granules in the endosperm and the pollen which stain red with iodine and which contain nearly 100% amylopectin; however, starch granules in other plant tissues contain both amylose and amylopectin and stain blue with iodine (*210*). Red-staining starches have been reported in other plant species, but these have not been characterized (*210*). Floridian starch found in the red algae also resembles amylopectin and lacks amylose (*10*, *16*).

Phenotypically, *wx* kernels are full and often appear opaque (Table IV) (*198*, *210*, *211*). Starch and dry weight production in *wx* kernels are equal to that in *normal* kernels and increase at similar rates (*41*, *44*, *87*, *96*, *212*, *213*). Sugar and WSP (Table VI) levels are also similar to *normal* in immature (*204*, *208*) and mature kernels (*205*).

The *wx* mutant is
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Genotype	*Gene expressed*[b]
Normal	None
wx	*wx*
ae	*ae*
su	*su*
su 2	*su 2*
du	*du*
ae wx	C
ae su	C
ae su 2	*ae*
ae du	C
du su	*su*
du su 2	C
du wx	C
su wx	*su*
su 2 wx	*wx*
su su 2	*su*
ae du su	*su*
ae du su 2	C
ae du wx	C
ae su su 2	C
ae su wx	C
ae su 2 wx	C
du su su 2	*su*
du su wx	*su*
du su 2 wx	C
su su 2 wx	*su*
ae du su wx	C

[a] Adapted from Garw
[b] If one gene is respo
expression giving a ne
individual genes.
[c] Kernels approach fu
[d] S.C. means the phe

The *wx* mutant is epistatic to all other known mutants relative to the lack of accumulation of amylose (*4*, *71*). Multiple mutants containing *wx* and *ae* have been reported to produce amylose (Table VII); but as will be pointed out in discussing the *ae wx* genotype, this apparent amylose, as measured by iodine

Table IV

Mature Kernel Phenotype of Normal and Selected Single, Double, Triple, and Quadruple Recessive Maize Genotypes[a]

Genotype	*Gene expressed*[b]	*Kernel phenotype*[c]
Normal	None	Translucent
wx	*wx*	Opaque
ae	*ae*	Tarnished, translucent, or opaque; sometimes semi-full
su	*su*	Wrinkled, glassy; S.C.[d]: Not as extreme
su 2	*su 2*	Slightly tarnished, often etched at base
du	*du*	Opaque to tarnished; S.C.: Semi-collapsed, translucent with some opaque sectors
ae wx	C	Semi-full to collapsed, translucent or glassy, may have opaque caps; S.C.: Slightly fuller, etched, translucent to glassy
ae su	C	Not quite as full as *ae*, translucent (tarnished in S.C.), may have opaque caps
ae su 2	*ae*	Translucent or opaque, etched base
ae du	C	Translucent, not as full as *ae*; S.C.: Etched, translucent, or tarnished
du su	*su*	Wrinkled, glassy (Duller than *su*); S.C.: Extremely wrinkled, glassy
du su 2	C	Translucent, etched
du wx	C	Semi-collapsed, opaque; S.C.: Shrunken, opaque
su wx	*su*	Wrinkled, glassy to opaque
su 2 wx	*wx*	Opaque, often etched
su su 2	*su*	Wrinkled, glassy
ae du su	*su*	Wrinkled, translucent; S.C.: Slightly wrinkled, translucent
ae du su 2	C	Semi-collapsed, translucent
ae du wx	C	Shrunken, opaque to tarnished; S.C.: Semi-collapsed, tarnished
ae su su 2	C	Partially wrinkled, translucent to tarnished
ae su wx	C	Semi-collapsed, opaque to translucent; S.C.: Etched, semi-full, translucent
ae su 2 wx	C	Etched, semi-full or wrinkled, translucent
du su su 2	*su*	Wrinkled, glassy
du su wx	*su*	Wrinkled, glassy
du su 2 wx	C	Semi-collapsed, opaque, etched
su su 2 wx	*su*	Wrinkled, glassy
ae du su wx	C	S.C.: Etched, semi-full, translucent to tarnished

[a] Adapted from Garwood and Creech (*198*).

[b] If one gene is responsible for the phenotype, that gene is listed. "C" signifies a complementary expression giving a new phenotype differing from the phenotypes of the stocks possessing the individual genes.

[c] Kernels approach full size unless indicated as semi-full, semi-collapsed, shrunken, or wrinkled.

[d] S.C. means the phenotype observed in sweet corn inbreds.

Table V

Mean Starch Granule Size, Birefringence End-Point Temperature (BEPT), and X-Ray Diffraction Pattern of 14 Maize Genotypes 24 Days Postpollination[a]

Genotype	Granule size, μm		BEPT	X-Ray pattern
	Minimum	Maximum		
Normal	7.99 fgh	8.53 jkl	70.3 bcdef	A
wx	8.61 gh	9.41 l	74.3 ef	A
ae	5.56 bcd	6.32 defg	97.7 h	B
su	3.06 a	3.52 a	69.0 abcde	A
su 2	7.68 fg	9.14 kl	63.7 a	A
du	5.19 bcd	5.98 cdef	70.7 bcdef	A
ae wx	5.67 bcd	6.03 cdef	82.3 g	B
ae su	5.34 bcd	8.20 ijk	88.0 g	B
ae su 2	5.57 bcd	6.46 defg	87.7 g	B
ae du	5.42 bcd	6.54 efgh	73.3 def	B
du su	3.11 a	3.85 ab	73.7 def	A[b]
du su 2	5.97 cde	8.79 jkl	63.3 a	A
du wx	6.18 de	6.86 fgh	76.7 f	A
su su 2	2.56 a	2.98 a	67.3 abcd	—

[a] Adapted from Brown and co-workers (*199*). Means followed by the same letter are not significantly different at the 1% level using Duncan's Multiple Range Test.

[b] Weak crystalline A pattern.

binding, is due to a loosely branched polysaccharide having long external chains (*69, 70, 214*). Owing to the lack of amylose, *wx* granules stain reddish-purple with iodine, although some *wx* granules have blue-staining cores (*1, 207, 210*).

Starch granules from maize, sorghum, and rice kernels homozygous for *wx* have been reported to have from 0–6% amylose (*82, 86, 87, 91, 94, 215*). This apparent amylose content may be due to the measurement technique used, to the effect of non-waxy starch granules from maternal tissue, to differences in the degree of branching, or to the presence of some linear material as suggested by blue-staining cores. If present, this linear material is minimal, for no amylose peak is observed in chromatographic profiles of *wx* starch (*69, 70, 71, 216*). These differences in apparent amylose content involve both cultivar and environmental effects as previously described for *normal* starch (*82, 86, 87, 91, 94, 215*). Alleles at the *wx* locus can also vary in amylose percentage with *wx-a* having 2–5% amylose compared to 0% in *wx-Ref* (*217, 218*). *Waxy* amylopectins vary in β-amylolysis limit, average chain length, and molecular size (*94, 219, 220*) as previously described for *normal* starch. The *normal* (*Wx*) allele is

Table VI

Water-Soluble Polysaccharide (WSP) Concentration in Immature and Mature Kernels of 26 Maize Genotypes[a]

	Immature		*Mature*
Genotype	*10% ETOH*[b]	*$HgCl_2$*[c]	*10% ETOH*[d]
Normal	3	0	4
wx	3	0	7
ae	4	0	6
su	28	25	19
su 2	2	—[e]	4
du	2	0	5
ae wx	6	0	5
ae su	4	0	5
ae su 2	4	—	6
ae du	7	0	5
du su	29	18	18
du su 2	3	—	4
du wx	11	2	8
su wx	29	19	17
su 2 wx	3	—	5
su su 2	31	—	19
ae du su	16	7	9
ae du su 2	10	—	8
ae du wx	4	Trace	6
ae su su 2	11	—	9
ae su wx	12	7	10
ae su 2 wx	6	—	7
du su su 2	35	—	32
du su wx	38	28	30
du su 2 wx	14	—	8
su su 2 wx	39	—	18

[a] All mutants were in a genetic background related to the single cross W23 × L317. Data expressed as percentage of kernel dry weight.

[b] Data adapted from Creech (*204*). WSP extracted with 10% ethanol.

[c] Data adapted from Black and co-workers (*13*). WSP extracted with aqueous $HgCl_2$; an aliquot hydrolyzed with H_2SO_4 and the increase in reducing sugar determined.

[d] Data adapted from Creech and McArdle (*205*). WSP extracted with 10% ethanol.

[e] Genotype not included in study.

Table VII

Apparent Amylose Percentages of Various Maize Genotypes Determined Using Iodine Binding Procedures[a]

Genotype	Reference		
	Kramer et al. (206)	*Seckinger and Wolf (207)*	*Holder et al. (208)*
Normal	27	27	29
wx	0	—[b]	<1
ae	61	57	60
su	29	—	33
su 2	40	28	38
du	38	35	34
ae wx	15	26	26
ae su	60	—	51
ae su 2	54	45	56
ae du	57	50	45
du su	63	13	40
du su 2	47	—	46
du wx	0	—	2
su wx	0	—	0
su 2 wx	0	—	0
su su 2	55	30	41
ae du su	41	—	28
ae du su 2	48	23	37
ae du wx	—	—	2
ae su su 2	54	—	31
ae su wx	13	4	14
ae su 2 wx	—	28	28
du su su 2	73	—	44
du su wx	0	—	0
du su 2 wx	0	—	0
su su 2 wx	0	—	0

[a] Colorimetric measurement of starch–iodine complex used to estimate apparent amylose percentages. Genotypes were not incorporated into an isogenic background.

[b] Genotype not included in study.

not completely dominant to the *wx* allele, and amylose percentage is reduced by several percent in the *Wx wx wx* endosperm genotype (*96, 221–224*).

The increase in average granule size during kernel development of *wx* maize and the final granule morphology of *wx* granules are similar to that of *normal* (*41, 199*). Also, as reported for *normal*, the average size of *wx* granules varies with the cultivar (*86, 94, 215*) and environmental conditions (*215*). Birefringence of *normal* and *wx* granules is similar; however, iodine staining reduces the

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Genotyp

Normal
wx
ae
su
du
ae wx
ae su
ae du
du su
du wx
su wx
ae du su
ae du wx
ae su wx
du su wx
ae du su wx
su phytoglycogen
Amylose–amylopectin

[a] Maize genotypes
Yeh and co-workers